Understanding enzymic binding affinity

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CHAPTER 5

Binding of Benzamidinium Chloride Inhibitors to Trypsin: Influence of the Dehydration of the Catalytic Triad by p-Alkyl Substituents

The effect of binding of p-alkylbenzamidinium chloride inhibitors to the serine proteinase trypsin on the hydration of the catalytic triad was studied using isothermal titration calorimetry and molecular dynamics simulation techniques. For p-n-hexylbenzamidinium at pH 7.4, a transfer of protons from the enzyme to the buffer was found that is, within error limits, equal to the number of protons calculated to be transferred upon binding of a natural protein inhibitor and larger than that found for unsubstituted benzamidinium chloride at pH 7.4. Since this proton transfer reflects the penalty of dehydrating His 57, these data indicate that His57 is more shielded from water upon binding of p-n-hexylbenzamidinium chloride than upon binding of benzamidinium chloride. The calculations corroborated that the p-alkyl substituents shield the active site from water: the more sterically demanding the substituent, the less water molecules are found in the surroundings of His57 and Ser195 and the larger the propensity to form a hydrogen bond between these two residues. This dehydration is found to be an important factor in determining the binding affinity. The results of this work suggest that the p-n-hexylbenzamidinium ion is a satisfactory model for the natural inhibitor of trypsin.

> "Aber auch die Bindungsregion des Trypsins ist nicht ganz starr. Die Seitenkette des Ser 195 weicht dem Inhibitorsegment aus, sein O^{γ} verschiebt sich dabei um 1,3 Å, so dass erst im Komplex eine kurze (2,7 Å) und nahezu lineare Wasserstoffbrücke zum $N^{\varepsilon 2}$ des His 57 möglich wird."
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5.1 Introduction

In the previous chapter it was demonstrated that sterically demanding substituents impose a thermodynamic cost on the binding of *p*-alkylbenzamidinium chlorides to trypsin. Here, an attempt will be made to determine the nature of that penalty. It is known that dehydration of the catalytic triad induced by binding of several natural protein inhibitors to serine proteinases induces an acidic shift in the pK_a of His57 N2.[2,3] Shifts in pK_a are an important source of information about neighbouring charges and dipoles, dielectrical permittivity and hydration of the environment of the α-amino acid.[4] This particular pK_a shift is attributed to the dehydration penalty of burying a charged His57 in a more apolar environment, which is partly compensated by a stronger Ser195-His57 hydrogen bond in the complex.[2,3] Below, the nature and implications of this shift will be considered.

For a variety of natural protein inhibitors, such as bovine pancreatic trypsin inhibitor (BPTI), binding to their cognate serine proteinases is less strong at lower pH.[3,5-10] Also, for the binding of turkey ovomucoid third domain[2] to elastase and Bowman-Birk inhibitor[5] to trypsin and chymotrypsin, the enthalpy of binding is less favourable at lower pH. From these pH-dependencies, a pK_a shift upon binding of the protein inhibitor from near 7 to near 5 can be deduced for all serine proteinases studied.[2,3,6-10] This acidic pK_a shift, attributed to His57 N2 in the active site, leads to a lower fraction of protonated His57 N2 in the complex. This is important for the catalytic machinery, since the uptake of a proton from the hydroxy group of Ser195 by His57 N2, crucial for the activation of Ser 195, is not possible for an already protonated nitrogen. At each specific pH, the change in the fraction of protonated His57 N2 upon inhibitor binding is the difference of the fractions for both pK_a's. These can be calculated using the Henderson-Hasselbalch equation:[11]

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\mathrm{His57\ N2}]}{[\mathrm{His57\ N2^+}]} \tag{5.1}$$

in a rewritten form:

$$\mathrm{His57\ N2^+} = \frac{1}{1+10^{\mathrm{pH\text{-}p}K_a}} \tag{5.2}$$

where [His57 N2] is the concentration of unprotonated His57 N2, [His57 N2$^+$] is the concentration of protonated His57 N2 and His57 N2$^+$ is the fraction of protonated His57 N2.

The pK_a shift of His57 N2 in a serine proteinase upon binding a protein inhibitor is thought to reflect changes in the active site that are induced by binding of the inhibitor.[2,3] The active site is largely shielded from water upon inhibitor binding.[2,3,10-15] Transferring a charge from water to a more apolar environment, such as an enzyme interior, is a very unfavourable process (the Gibbs energy of transfer is around 40 kJ mol^{-1}).[16] Since the fraction of protonated His57 in free trypsin is higher at low pH, upon binding more protons will be transferred to the buffer at low pH. This is an

unfavourable process too, although less unfavourable than burying a charge, and therefore the binding constant is lower at low pH.

However, if dehydration were the only mechanism responsible for the pK_a shift, the shift would be much more acidic.[2] A difference in Gibbs energy of 40 kJ mol^{-1} correlates with a pK_a shift of 7 units, indicating that other effects must be counteracting the dehydration process and thereby stabilise the positive charge. It is known that Ser195 OG reorientates upon binding of the inhibitor due to a rotation of the hydroxy group around the carbon-carbon bond.[3,10,12,14,15] In the dehydrated complex, Ser195 OG cannot make, or at least, to a lesser extent, a hydrogen bond to water anymore and His57 N2 is almost completely deprotonated. Therefore, the hydrogen bond to His57 N2 will become stronger.[17] The stronger hydrogen bond facilitates proton transfer from Ser195 OG to His57 N2, thereby increasing the nucleophilicity of Ser195 OG.[18] The catalytic triad does not exist in the free enzyme, since the Ser195-His57 hydrogen bond is not formed.[14] Since protein inhibitors bind in a manner similar to the pretransition state complex of a peptide substrate,[11,13,14,19,20] it can be argued that binding of the substrate activates the catalytic triad of the enzyme by increasing the nucleophilicity of Ser195 OG due to a lower fraction of protonated His57 N2 that is accompanied by a stronger His57 N2-Ser195 OG hydrogen bond. The formation of this hydrogen bond upon complexation with substrates, allowing the enzyme to become catalytically active, has also been corroborated by molecular dynamics simulations of the conformation of the active site of porcine pancreatic elastase.[21] In the hydrated native enzyme this hydrogen bond is not present, due to competition for the acceptor atom His57 N2 between water molecules and Ser195 OG. In the Michaelis complex with the hexapeptide Thr-Pro-nVal-Leu-Tyr-Thr, this hydrogen bond is more readily formed.

Crystallographic data of Marquart et al.[15] also show that the His57 N2-Ser195 OG hydrogen bond becomes stronger in the complexes due to a reorientation of the hydroxy side chain of Ser195 OG that considerably improves the bond length and angle of this hydrogen bond, whereas the interaction with an internal water molecule becomes weaker or even disappears. Figure 5.1 shows the His57-Ser195 active site base pair and the interaction with the internal water molecule 702. For the distance His57 N2-Ser195 OG, in respectively free trypsin, the benzamidinium-trypsin complex and the natural BPTI-trypsin complex, values of 3.1, 3.0 and 2.6 Å were reported. The Ser195 OG-Sol702 distance is 2.9 Å in free trypsin and 3.3 Å in benzamidinium-trypsin complex, whereas in the BPTI-trypsin complex, this water molecule is absent. The inverse correlation between these two distances shows the influence of Sol 702 on the hydrogen bond between Ser195 and His 57. The angle His57 N2-Ser195 OG-CB (α, Figure 5.1) reported for free trypsin, the benzamidinium-trypsin complex and the BPTI-trypsin complex are 83.7, 87.7 and 103.2° respectively. A value of 111° is expected for a perfectly linear hydrogen bond.

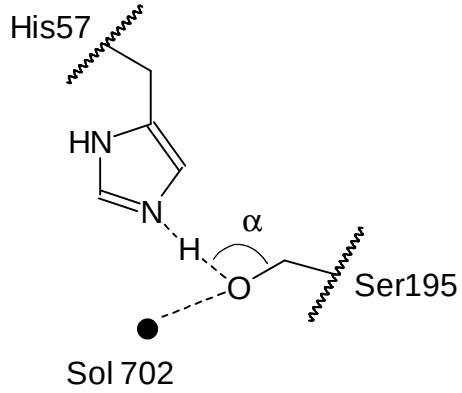

Figure 5.1. *Schematical representation of the Ser195-His57 active site base pair, together with the internal water molecule 702; distances between His57 N2-Ser195 OG and Ser195 OG-Sol702, as well as the angle His57 N2-Ser195 OG-CB (α) for free trypsin, trypsin-benzamidinium complex and BPTI-trypsin complex are listed in the text.*

These data indicate that whereas binding of benzamidinium has only a moderate effect on the conformation of the catalytic triad, binding of BPTI induces an almost perfect, linear hydrogen bond. In the BPTI-trypsin complex, the active site is completely shielded from water, whereas in the benzamidinium-trypsin complex some water is still present.[15] In the BPTI-trypsin complex, the carbonyl carbon of what would be the "scissile" bond in substrates is, with a distance of 2.7 Å, in closer than van der Waals contact with Ser195 OG.[14,15] Due to the rigidity and the tightness of the complex water cannot enter the reaction site.[11] In the benzamidinium-trypsin complex, on the other hand, the distance between Ser195 OG and the hydrogen on the *p*-position of the benzamidinium, is larger.[22] We expect the effect of a substituent on the *p*-position of a benzamidinium inhibitor to be larger for more sterically demanding substituents, since the shielding from water of Ser195 OG would be larger in that case.

In this study,[1] we investigated the buffer dependence of the thermodynamics of binding of benzamidinium chloride and *p*-*n*-hexylbenzamidinium chloride to trypsin at pH 7.4 by means of isothermal titration calorimetry (ITC). This dependence has been used to deduce the change in the fraction of protonated His57 N2, yielding valuable information on the hydration of the catalytic triad. Furthermore, molecular dynamics (MD) simulations have been used to study the influence of binding of *p*-*n*-alkylbenzamidinium chlorides on the hydration and the conformation of the catalytic triad.

5.2 Buffer Dependence of Binding of Benzamidinium Chloride and *p-n*-Hexylbenzamidinium Chloride to Trypsin

In the introduction, the pK_a shift upon binding of natural inhibitors to serine proteinases was described. To examine whether a change in the protonation state of His57 N2 also occurs upon binding of the benzamidinium-based inhibitors to trypsin, we have investigated the dependence of binding of benzamidinium chloride and *p-n*-hexylbenzamidinium chloride on the buffer used. When protonation/deprotonation effects are significant in the binding process, the observed enthalpy of binding ΔH not only consists of the intrinsic enthalpy of binding (ΔH_{int}) but also depends on the enthalpy change associated with ionisation of the buffer (Section 2.2.1).[23-25] This can be expressed as:

$$\Delta H = \Delta H_{int} + N_{H^+} \Delta H_{ion} \tag{5.3}$$

where N_{H^+} is the number of protons from the buffer taken up by the enzyme and ΔH_{ion} is the ionisation enthalpy of the buffer. N_{H^+} is equal to the change in the protonation state of residues involved in the binding process.

Table 5.1. *Thermodynamic parameters for binding of benzamidinium chloride and p-n-hexyl-benzamidinium chloride to trypsin. Titrations performed in 50 mM (10 mM $CaCl_2$) of different buffers at either pH 8.0 or 7.4 at 25 °C.*

R[a] (pH)	buffer	K (10^4 M^{-1})	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})
H (8.0)	Hepes	5.3	-26.9	-17.9[b]	9.0
	Bicine	6.3	-27.4	-16.0[b]	11.4
	Tricine	4.6	-26.6	-16.8[b]	9.8
	Glycylglycine	5.0	-26.8	-16.5[b]	10.3
	Tris[b]	4.5	-26.6	-18.9	7.7
H (7.4)	Pipes	4.6	-26.6	-13.2	13.4
	Hepes	4.9	-26.8	-15.3	11.5
	Bicine	4.3	-26.5	-15.7	10.8
	Tricine	3.8	-26.1	-16.5	9.6
n-Hex (7.4)	Pipes	15	-29.5	-2.9	26.6
	Hepes	14	-29.4	-6.2	23.2
	Bicine	13	-29.2	-6.6	22.6
	Tricine	12	-29.1	-8.4	20.7

[a] *Substituent at p-position.* [b] *From Chapter 2.*

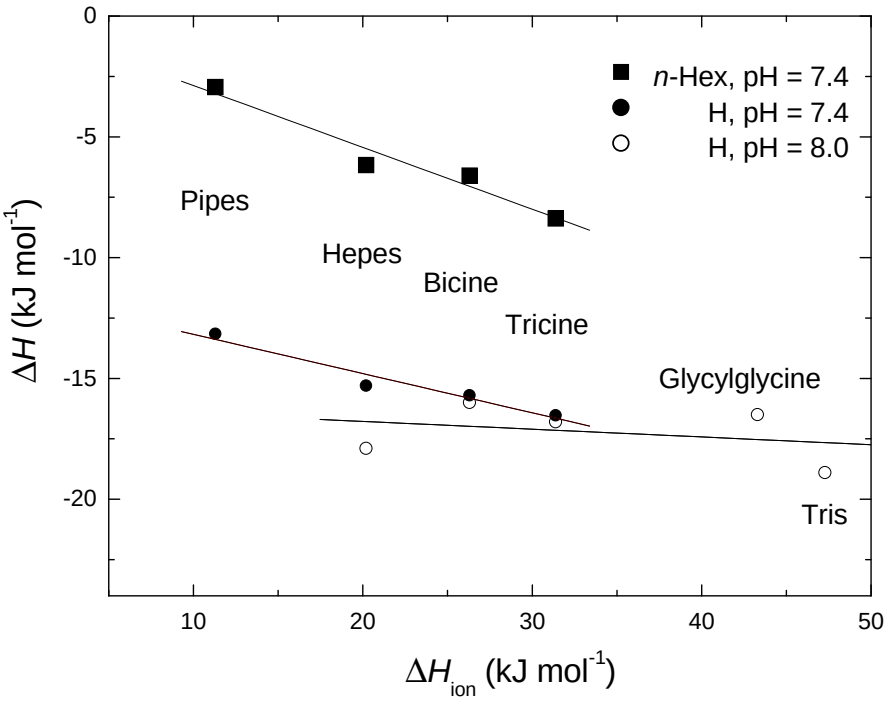

Figure 5.2. *Enthalpy of binding of benzamidinium chloride at pH 8.0 and 7.4 and of p-n-hexylbenzamidinium chloride at pH 7.4 versus the ionisation enthalpy[26] of the buffer.*

Table 5.1 lists the thermodynamic parameters of binding of benzamidinium chloride to trypsin at 25 °C in several buffers at pH 8.0 and of both benzamidinium chloride and *p-n*-hexylbenzamidinium chloride at pH 7.4. Figure 5.2 shows the dependence of the observed enthalpy of binding on the ionisation enthalpy of the buffer for benzamidinium chloride at pH 8.0 and 7.4 and *p-n*-hexylbenzamidinium chloride at pH 7.4, all at 25 °C.

For benzamidinium chloride at pH 8.0, the linear regression of the data yields an abscissa, equal to ΔH_{int} (Eq. 5.3), of –16.3 (1.9) kJ mol^{-1} and a slope, equal to N_{H^+} (Eq. 5.3), of –0.03 (0.05) indicating that, within error limits, no protons are transferred upon binding. The mean value of the binding constants (K_{mean}) listed in Table 5.1, is $5.1 \cdot 10^4$ M^{-1}, corresponding to a ΔG_{mean} of –26.9 kJ mol^{-1}. From ΔG_{mean} and ΔH_{int}, a value for $T\Delta S_{int}$ of 10.9 kJ mol^{-1} is calculated.

Next, we look at the influence of lowering the pH. For benzamidinium chloride at pH 7.4, the values of ΔH_{int} and N_{H^+} are -11.6 (0.6) kJ mol^{-1} and –0.16 (0.03), respectively. Using the data in Table 5.1, K_{mean} is calculated to be $4.4 \cdot 10^4$ M^{-1} and ΔG_{mean} is -26.5 kJ mol^{-1}, leading to $T\Delta S_{int}$ is 14.9 kJ mol^{-1}. Comparing the data at pH 7.4 with the data at pH 8.0, we can conclude that at pH 7.4 more protons are transferred upon binding. Furthermore, ΔG_{mean} is lower, due to a less favourable ΔH_{int} that is partly compensated by a more favourable $T\Delta S_{int}$. Thus, similar to natural protein inhibitors, at

lower pH more protons are transferred upon binding and the binding constant is decreased due to a less favourable ΔH_{int} that is partly compensated by a more favourable entropic contribution.

It is interesting to look at the influence of an alkyl chain at the *p*-position on the thermodynamics of binding and N_{H^+}. For *p*-*n*-hexylbenzamidinium chloride at pH 7.4, the fitted values of ΔH_{int} and N_{H^+} are -0.3 (0.9) kJ mol^{-1} and -0.26 (0.04), respectively. K_{mean} is 1.4·10^5 M^{-1} and ΔG_{mean} is -29.3 kJ mol^{-1}, leading to a value for $T\Delta S_{int}$ of 29 kJ mol^{-1}. The intrinsic enthalpy of binding is close to zero, indicating that the driving force for binding is almost entirely entropic.

It is evident that at pH 7.4 more protons are transferred upon binding for *p*-*n*-hexylbenzamidinium chloride than for benzamidinium chloride. In the introduction it was mentioned that for serine proteinases a pK_a shift of His57 from 7 to 5 occurs upon binding a protein inhibitor. Using Eq. 5.2, it can be calculated that at pH 7.4 the fraction of protonated His 57 in a free serine proteinase, with pK_a (His57) = 7, amounts to 0.285, whereas it amounts to 0.004 when a natural protein inhibitor is bound, with pK_a (His57) = 5. Therefore, the change in the fraction of protonated His57 N2 for a serine proteinase upon binding of a natural protein inhibitor at pH 7.4 is calculated to be –0.28 (the difference between both fractions: 0.004 – 0.285). This decrease in the fraction of protonated His57 N2 corresponds to a transfer of protons from the enzyme to the buffer of 0.28, or from the buffer to the enzyme (N_{H^+}) of -0.28.[27] This value is, within error limits, equal to the value reported for the binding of *p*-*n*-hexylbenzamidinium chloride to trypsin and larger than that reported for benzamidinium chloride (see above). These results are in line with our expectations that the effect of a substituent on the *p*-position of a benzamidinium inhibitor is larger for more sterically demanding substituents. We therefore contend that the measured proton transfer upon binding of those two inhibitors is caused, similar to natural protein inhibitors, by the acidic pK_a shift of His57 N2. Since the decrease of the fraction of protonated His57 most likely reflects a dehydration penalty, these data suggest that His57 is more shielded from water upon binding of *p*-*n*-hexylbenzamidinium chloride than upon binding of benzamidinium chloride.

5.3 Influence of Binding of *p*-Alkylbenzamidinium Ions on the Hydration and Hydrogen Bond Formation Between Ser195 OG and His57 N2

In Section 5.1 it was argued that the acidic pK_a shift of His57 N2 upon inhibitor binding is the result of the dehydration of this residue in the complex and the reorientation of the hydroxy group of Ser195. Here, the results of the MD simulations of the benzamidinium- and *p*-alkylbenzamidinium-trypsin complexes, described in Chapter 4, are used to test the hypothesis that more sterically demanding substituents are more efficient in shielding the catalytic triad from water and inducing the hydrogen bond between Ser195 OG and His57 N2.

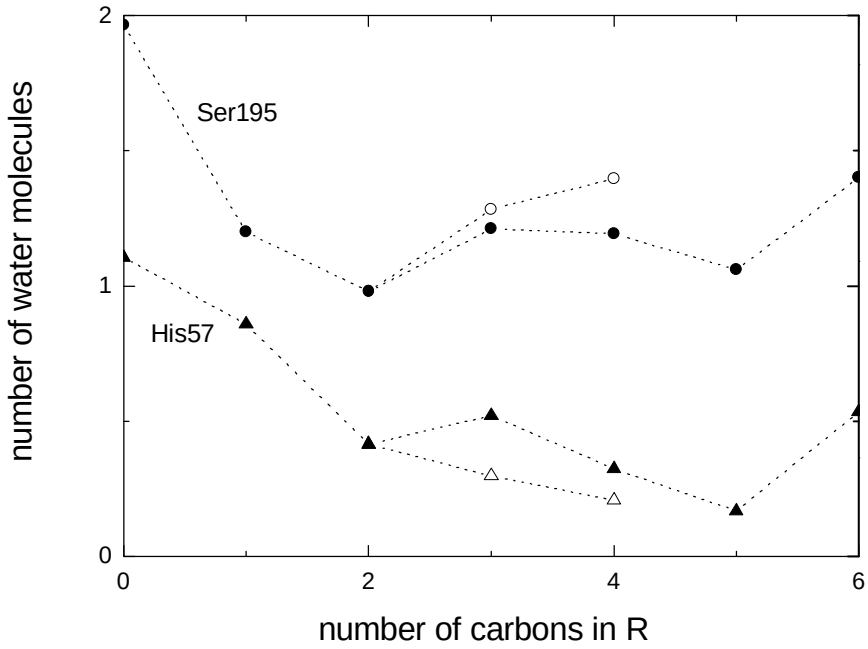

Figure 5.3. *Average number of water molecules in a radius of 3.5 Å around Ser195 OG and His57 N2 in p-alkylbenzamidinium-trypsin complexes. Open symbols are i-Pro and t-Bu, closed symbols are linear tails.*

In Figure 5.3, the average number of water molecules around Ser195 OG and His57 N2 in the modelled complexes is plotted versus the number of carbon atoms in the *p*-alkyl substituent. Upon increasing this number of carbon atoms, the two residues (in particular His57) become progressively more shielded and therefore less accessible to water. This trend seems to reverse when a tail length of six carbons (*p*-*n*-hexyl) is reached.

A relation can be observed between the average number of water molecules around Ser195 OG and His57 N2 (Figure 5.3) and the distribution of the distance between Ser195 and His57. The distance between Ser195 OG and His57 N2 in the crystal structure of the benzamidinium-trypsin complex is 3.0 Å.[15] A hydrogen-bond distance between these two residues allows the uptake of a proton from the hydroxyl group of Ser195 by His57 N2, which is a crucial step for the catalytic mechanism of the enzyme. In the simulation, we observe an average distance of 3.3 ± 0.1 Å, a value close to the crystal structure data. Figure 5.4 shows the distribution of this distance between Ser195 OG and His57 N2 for the complexes of trypsin with the *p*-methyl, *p*-*i*-propyl and *p*-*n*-hexylbenzamidinium ions. The distributions for the other complexes are between *p*-methyl and *p*-*n*-hexyl for the linear chains (except for *p*-*n*-pentyl which is similar to *p*-*i*-propyl) or between *p*-methyl and *p*-*i*-propyl for branched groups.

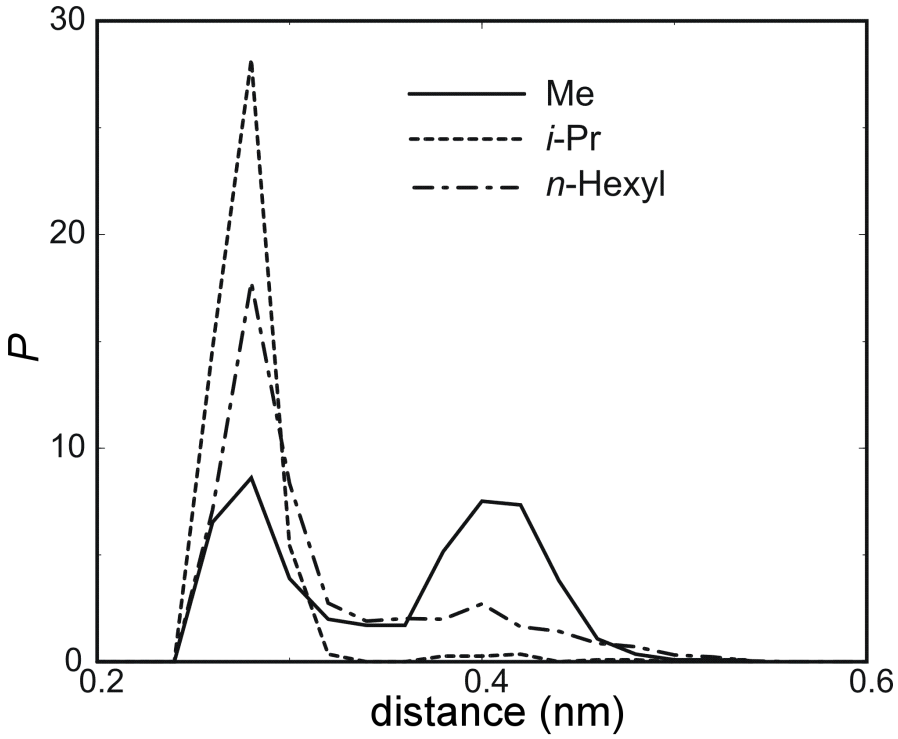

Figure 5.4. *Distribution functions of the distance (nm) between the oxygen OG of Ser195 and the nitrogen N2 of His57 in the complexes of trypsin with p-methyl-, p-i-propyl-, and p-n-hexyl-benzamidinium ions.*

In these distributions two maxima can be observed: one at 2.8 Å, corresponding to a hydrogen bond distance and the other around 4.0 Å. The higher the branching on the first carbon, the higher the chance that a hydrogen bond is formed between the two residues. In fact, *i*-propyl- and *t*-butyl-benzamidinium assure the presence of a hydrogen bond between Ser195 and His57 (Figure 5.4). The probability of a hydrogen bond in the catalytic triad also increases upon elongation of the carbon tail (Figure 5.4). It might be expected that the shielding of the catalytic triad and thus the formation of a hydrogen bond depends on the position of the tail relative to the catalytic triad. However, no relation has been observed between the movement of the tail (Section 4.2.3) and the formation and breaking of hydrogen bonds between Ser195 and His57.

It is concluded that exclusion of water from the active site promotes the formation of a hydrogen bond between the OG of Ser195 and the N2 of His57. The substituent at the *p*-position shields the active site from water: the more sterically demanding a substituent is, the less water can be found around Ser195 OG and His57 N2 and the larger the propensity to form a hydrogen bond between these two amino acid residues. This dehydration of the catalytic triad upon binding induces an acidic pK_a shift of His57 N2 (Section 5.1). These results corroborate our suggestion (Section 5.2) that the measured proton transfers are due to the shielding of His57 N2 from water and that this shielding is larger for *p*-*n*-hexylbenzamidinium chloride than for benzamidinium chloride. The dehydration of the catalytic triad also involves a thermodynamic cost and therefore results in a lower

binding affinity. In Table 4.3, it can be seen that for the structural variations with $\Delta\Delta G$ larger than zero, i.e. for unfavourable mutations, $\Delta\Delta H$ is unfavourable and not completely compensated by a favourable $\Delta(T\Delta S)$. For the favourable mutations, the more favourable entropic contribution does outweigh the change in the enthalpic contribution. In most of these cases, the change in the enthalpic contribution is unfavourable, except for the longer tails, where $\Delta\Delta H$ is zero for the mutation from *n*-butyl to *n*-pentyl and favourable for the mutation from *n*-pentyl to *n*-hexyl. It therefore appears, as anticipated in Chapter 1, that specific interactions of bound water molecules with the catalytic triad of trypsin may add a favourable enthalpic component that outweighs the entropic cost of immobilising this water molecule. At the risk of overinterpreting our results, we propose that the fact that upon going from *p*-*n*-pentylbenzamidinium to *p*-*n*-hexylbenzamidinium, ΔG decreases more than according to the trend for the smaller substituents (Table 4.1 and Figure 4.2A), is partly due to the fact that dehydration also becomes less extensive upon that one-methylene increment of the tail length of the inhibitor (Figure 5.3). This decreased dehydration may be coupled to the fact that for *p*-*n*-hexylbenzamidinium, in contrast to *p*-*n*-butylbenzamidinium, the conformation where the tail is bound in the S3/S4 pocket dominates, which might disable the tail to shield the catalytic triad.

The association of inhibitor binding with the dehydration of the active site and the accompanying changes in the interaction between Ser195 and His57 at least partially explain the apparent discrepancy between the experimental relative binding Gibbs energies and those calculated based on the MD simulations and on the *n*-octanol-water partition constants[28] (Figure 4.5). The relative binding affinities based on the *n*-octanol-water partition constants are too large, since in that case the dehydration of the protein is not taken into account. The effect of dehydration of the protein is expected to be reflected in the free-energy calculations. Indeed, it has been recently shown that the force field used in the calculations reproduces, with high accuracy, the cyclohexane/water partition properties of small linear and branched aliphatic compounds. In contrast, the force field was shown to underestimate the Gibbs energy of hydration of analogues of the more polar α-amino acids including Trp.[29] Therefore, the calculations are expected to correctly reproduce the hydrophobic contributions to binding and to underestimate the cost of dehydration of the protein. This would lead to an overestimation of the calculated binding Gibbs energies, as is indeed seen to be the case in Figure 4.5. Thus, the free-energy calculations reinforce the idea that dehydration is an important determining factor for binding. It is also interesting to note that the degree of dehydration of the catalytic triad is influenced by both the length of the substituent and the degree of branching at the first carbon (Figure 5.3). For example, for the transformation of ethyl to *i*-propyl it is not possible to distinguish steric effects due to clashes with the protein from effects due to the increased dehydration cost of the active site.

5.4 Conclusions

For the binding of *p*-*n*-hexylbenzamidinium to trypsin, a decrease in the fraction of protonated residues involved in binding has been observed that is, within error limits, the same as that calculated for binding of natural protein inhibitors. In the latter case, this decrease is caused by an acidic pK_a shift of His57 N2 due to dehydration of the catalytic triad. For the binding of benzamidinium to trypsin, a smaller decrease was found. The influence of the alkyl substituent on the surroundings of the catalytic triad has been probed using molecular dynamics simulations. The substituent was found to shield the active site from water: the more sterically demanding a substituent is, the less water can be found around Ser195 OG and His57 N2 and the larger the propensity to form a hydrogen bond between these two amino acid residues. This dehydration is thermodynamically unfavourable and therefore results in a lower binding affinity.

Protein inhibitors bind in a manner similar to a peptide substrate.[11] Mechanistically, the stronger hydrogen bond and the lower fraction of protonated His57 N2, induced by binding, serve to facilitate the uptake of the Ser195 OG proton by His57 N2 and thereby activate the catalytic triad of the enzyme. Apparently, due to its capability to effectively shield the active site from water, the small *p*-*n*-hexylbenzamidinium inhibitor is able to activate the catalytic triad to an extent similar to that of a protein inhibitor. Benzamidinium chloride is also capable of activating the catalytic triad, but to a much lesser extent, as evident from the smaller decrease in the fraction of protonated residues involved in binding. This is consistent with the crystal structure, which reveals some improvement of the His57-Ser195 hydrogen bond upon binding of benzamidinium to trypsin, though very modest in comparison to that for BPTI. Since benzamidinium is a model structure for the lysine and arginine side chains of natural protein inhibitors, we contend that an *n*-hexyl chain attached on its *p*-position is able to mimic the rest of the protein with respect to the activation of the catalytic triad. It would be interesting to determine the change in the fraction of protonated residues involved in binding of the inhibitors that are more sterically demanding, since these are even more efficient in shielding the active site from water (Figure 5.3) and thereby activating the catalytic triad (Figure 5.4).

5.5 Acknowledgements

Dr. Alessandra Villa is gratefully acknowledged for performing the MD simulations and for helpful discussions that were indispensable for the in-depth analysis of the influence of binding of the inhibitors on the catalytic triad.

5.6 Experimental Section

General remarks.

Bovine pancreatic trypsin was obtained from Fluka. Trypsin solutions were prepared as described in Section 2.7. Benzamidine hydrochloride was purchased from Sigma and *p-n*-hexylbenzamidinium chloride was synthesised as described in Section 4.6.

Isothermal titration calorimetry.

Titration experiments were performed as described in Section 2.7.

Computational Techniques.

The molecular dynamics simulations were performed as described in Section 4.6.

5.7 References and Notes

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